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Temperature and density dependent induction of a cytopathic effect following infection with non-cytopathic HAV strains

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ABSTRACT

Hepatitis A virus infection and growth in cultured cells is protracted, cell-type restricted, and generally not accompanied by the appearance of a cytopathic effect, with the exception of some culture-adapted strains. We demonstrate that the non-cytopathic HAV strain HM175/clone 1 can be induced to exhibit a cytopathic phenotype in both persistently or acutely infected cells under co-dependent conditions of lower incubation temperature ($<34\ ^{\circ}C$) and reduced cell density in both monkey (FRhK-4) and human (A549) cells. This phenotype is not virus-strain restricted, as it was also observed in cells infected with HAV strains, HAS-15 and LSH/S. Cytopathic effect was accompanied by rRNA cleavage, indicating activation of the RNase L pathway, viral negative strand synthesis, caspase-3 activation, and apoptosis. The results indicate that a cytopathic phenotype may be present in some HAV strains that can be induced under appropriate conditions, suggesting the potential for development of a plaque assay for this virus.

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Introduction

Hepatitis A virus (HAV) is a member of the picornavirus family of RNA viruses and currently the only member of the genus Hepatovirus (Norder et al., 2011). This virus remains the major causative agent of infectious hepatitis worldwide and is transmitted primarily via the fecal-oral route; its dissemination via person-to-person contact as well as contaminated food and water has been documented and is the subject of numerous reviews (Cliver, 2010; Fiore, 2004; Koopmans and Duizer, 2004). HAV is stable, relatively resistant to inactivation and decontamination methods often successful against non-enteric viruses, and can remain infectious under varying environmental conditions as well as on environmental surfaces for substantial periods of time.

Early research investigations achieved the ability to culture HAV derived either directly from human samples or subsequent to passage of human isolates in monkeys. For example, Provost and Hilleman (1979) demonstrated that primary cell cultures of monkey origin were permissive for HAV replication and supported the production of infectious virus (human isolate) post-passage in monkeys. Frösner et al. (1979) reported the use of an established cell culture line that was permissive to replication of HAV directly following virus extraction from an outbreak (human fecal) sample. Other investigators (Flehmig, 1980, 1981; Flehmig et al., 1981) studied the direct application of human stool samples containing HAV to a rapidly growing, established cell line without the requirement for additional agents such as the presence of HBV genome in the cell line used by Frösner et al. (1979). Subsequent work from these as well as other investigators revealed significant results regarding the culture adaptation of wild-type strains (Hollinger, 2001; Nainan et al., 2006; Ross et al., 1991). For example, HAV infection and growth in cultured cells is cell-type restricted; permissive replication is typically demonstrated in primate cells of non-human origin. However, the human cell line A549 and the embryonic diploid cells WI-38 and MRC-5 have also been shown to be permissive for at least some strains of HAV.

Following acute infection in culture, HAV replication is slow, requiring weeks/months for consistent detection of expressed viral antigen and/or particles without onset of a cytopathic effect and thus can establish a persistent (steady-state) infection. Serial passage/sub-culture of HAV strains can result in culture-adaptation of the virus typically defined by increased virus replication rates and expression of viral antigen, and has been achieved with various strains from human isolates as well as monkey passaged human virus (Binn et al., 1984; Bradley et al., 1984; Daemer et al., 1981; Flehmig, 1980; Flehmig et al., 1981; Frösner et al., 1979; Gauss-Müller et al., 1981; Kojima et al., 1981; Provost and Hilleman, 1979). Indeed, these persistently infected cell lines can be routinely sub-cultured, as with the uninfected parental line, often for up to a year without overt induction of apoptosis, cpe or dramatic changes in morphologic appearance, but with

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viral RNA, protein, and infectious virus recovery from these cells. The reason(s) for the protracted replication rate of wild-type virus and culture-adapted strains (despite the improved replication of the latter compared to the former) is not completely known; however, it has been posited that HAV, which lacks a host shut-off function typical of other picornaviruses, competes for critical cellular factors essential for virus gene expression and replication (Bedard and Semler, 2004; Pinto et al., 2007). Alternatively, it has been suggested that slow replication and virus particle maturation/yield may be the consequence of ongoing encapsidation of the viral RNA pool, asynchronous replication, down regulation of viral RNA replication or rate-limiting viral translation (Anderson et al., 1988; Cho and Ehrenfeld, 1991; De Chastonay and Siegl, 1987; Funkhouser et al., 1999).

The serial passage of cell-culture adapted non-cytopathic HAV strains has resulted in the emergence of even faster growing strains capable of inducing a cytopathic effect (cpe) during virus replication. Interestingly, the induction of cpe appears to be restricted to particular cell lines, for example FRhK-4 (and their derivative Frp/3), B-SC-1 and A549 cells (Ali, 2002; Anderson, 1987; Cromeans et al., 1989; Cromeans et al., 1987; Divizia et al., 1986; Gaspar et al., 1992; Lemon et al., 1991; Nasser and Metcalf, 1987; Venuti et al., 1985); the reason(s) for this cell restricted phenotype is not yet fully understood. In this regard, both non-cpe and cpe HAV strains remain of interest to investigators particularly those studying the processes responsible for viral replication and cpe induction by HAV and the contribution of mutations in the viral genome to these processes (Beneduce et al., 1995; Brack et al., 1998; Cohen et al., 1989; Emerson et al., 1991; Funkhouser et al., 1994; Jansen et al., 1988; Lemon et al., 1991). Induction of cpe is also of interest to investigators in providing a mechanism by which to quantitate viral replication. Given the non-cytopathic nature of wild-type and culture-adapted HAV strains, early investigators applied techniques such as radioimmunoassay (RIA), direct and indirect immunofluorescence, and immune electron microscopy to evaluate whether virus replication and gene expression occurred in HAV infected cells. The quantification/titration of HAV infectivity later included established methods such as radioimmunofocus assays (RIFA), immunofluorescence, ELISA, RT-PCR, immunological-based focus assays, and in situ hybridization (Jansen et al., 1985; Lemon et al., 1983; Richards and Watson, 2001; Siegl et al., 1984a; Yeh et al., 2008). Notably, a plaque assay has not been developed for either wild-type or culture-adapted HAV due to the absence of cytopathogenicity of these strains. However, there is a real need for a relatively simple quantitative methodology that does not involve radionuclide(s) and demonstrates both infectivity and production of infectious progeny, all features that a plaque assay would provide.

During the present investigation, we discovered that culture incubation temperature can affect the phenotype of a culture-adapted, non-cytopathic HAV strain (HM175/clone 1) during persistent infection. We then sought to determine what other incubation and infection parameters can affect the regulation and expression of this phenotype. Our results indicate that (i) culture incubation temperature in combination with cell culture density affect the temporal onset of cpe, (ii) cpe is the result of the induction of caspase-dependent apoptosis in persistently infected cells and is commensurate with caspase activation in acutely infected cells, (iii) development of cpe is not the result of an abortive infection as viral protein (capsid) and negative strand synthesis is readily detected during infection, and (iv) the induction of cpe occurs during acute infection with three culture-adapted, non-cytopathic HAV strains and, therefore, is not solely a viral strain restricted phenomenon. Interestingly, the induction of cpe/apoptosis in infected cells is preceded by rRNA cleavage (viz. activation of the OAS/RNase L pathway) and therefore may be dependent on the activation of the OAS protein. We postulate that other non-cytopathic HAV strains may also be similarly affected to induce apoptosis/cpe in a manner that is likely co-dependent on these and other factors such as virus replication rate, the presence/absence of critical cell regulatory factors and possibly the activation of the RNase L pathway.

Results

Induction of cpe phenotype in HM175/clone 1 infected cells during incubation at 33 °C

In our laboratory, the regular growth and maintenance of FRhK-4 (monkey) cells, as well as FRhK-4 cells persistently infected with HAV HM175/clone 1 (also called clone 1 cells (Kulka et al., 2009)) are typically conducted at an incubation temperature of 37 °C (Goswami et al., 2004; Kulka et al., 2009; Kulka et al., 2003). Clone 1 cells can be sub-cultured on a weekly basis up to 240 days post infection (dpi) without the appearance of cpe and preliminary studies in our laboratory have shown that continuous sub-culture for up to approximately 1 year pi is possible, albeit with subtle morphologic changes becoming increasingly visually evident as the period of sub-culture increases (beginning at approximately 250 days pi) (data not shown). During routine sub-culturing we discovered that incubation of persistently infected cells at 34 °C resulted in the onset of a "cpe-like" morphology after 7 days (data not shown). Continued investigation revealed that the induction of cpe was visually discernible beginning at approximately 3–4 days growth at either 33 °C or 34 °C (data not shown). As discussed in the Materials and Methods, we conducted further investigations at 33 °C unless otherwise stated. In Fig. 1, the effect of 33 °C incubation is shown for FRhK-4 cells either uninfected or persistently infected with HM175/clone 1 using brightfield microscopy (panels (A)–(C)). Persistently infected cells cultured at 37 °C (panel (B)) have a morphologic appearance similar to that observed for uninfected FRhK-4 cells cultured either at 37 °C (data not shown, Goswami et al., 2004) or 33 °C (panel (A)). When persistently infected cells are cultured at 33 °C, the development of cpe-like morphology is observed beginning at day 4 (data not shown). Both the number and the severity of cells exhibiting this morphology increases with increasing time of incubation at 33 °C and is shown for persistently infected cells at day 6 (panel (C)), and it is easily distinguishable from similarly sub-cultured cells incubated at 37 °C (panel (B)).

We were interested in determining whether the apparent temperature-dependent morphologic change observed in the infected FRhK-4 cells is restricted to a given cell-type. To this end we established an HM175/clone 1 persistently infected A549 (human) cell line as these cells have been reported previously to support the replication of HAV (Cromeans et al., 1989). As shown in Fig. 1, persistently infected A549 cells cultured at 37 °C (panel (E)) demonstrate little morphologic differences from uninfected A549 cells at either 37 °C (data not shown) or 33 °C (panel (D)). As with FRhK-4 cells, cpe-like morphology develops at approximately 4 days (data not shown) and increases with time of incubation at 33 °C and is readily apparent at day 6 (panel (F)), and it appears that both persistently infected cell lines grow more slowly at 33 °C than at 37 °C (data not shown). Importantly, the results indicate that induction of cpe is co-dependent on HAV infection and incubation temperature, and is not cell type restricted. Indeed, we believe that the induction of cpe during cell culture infection with a non-cytopathic strain of HAV is a significant discovery since as far as we are aware it has not been previously reported in the literature.

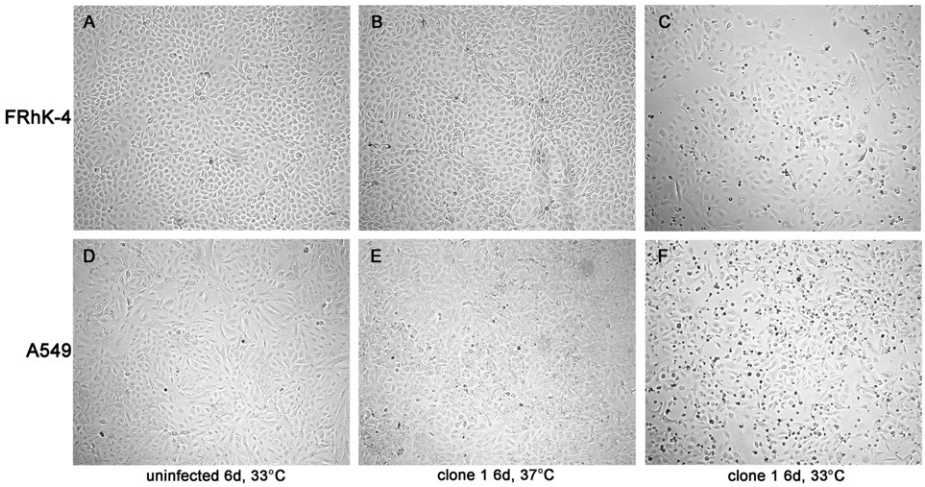

Fig. 1. Brightfield microscopy for the examination of HM175/clone 1 persistently infected or uninfected FRhK-4 or A549 cells following growth at either 33 °C or 37 °C. Uninfected and persistently infected FRhK-4 ((A)–(C)) and A549 ((D)–(F)) cells were seeded at 4000 and 6000 cells/cm^2, respectively, then cultured for 6 days at either 33 °C ((A), (C), (D), (F)) or 37 °C ((B), (E)). Cultures were observed over the 6 days incubation period by brightfield microscopy and images were obtained and shown for day 6 of the culture period.

Induction of rRNA cleavage in either FRhK-4 or A549 cells persistently infected with HM175/clone 1

We have previously shown that FRhK-4 cells are permissive for HAV replication and that rRNA cleavage in these cells can be detected as early as 16 h pi with HAV HM175/18f without prior treatment with IFN but cannot be detected in HAV HM175/clone 1 infected (acute or persistent) cells (Kulka et al., 2009; Kulka et al., 2003). We also established that the RNA cleavage in 18f infected cells was indicative of an activated RNase L since its characteristic pattern (and attribution to RNase L activation) was as described by others (Cayley et al., 1982; Kulka et al., 2009; Silverman et al., 1983; Wreschner et al., 1981) and its detection was commensurate with the presence of the well-characterized activator of RNase L, 2–5 A (Kulka et al., 2009).

Given the striking similarity between the cpe observed in Fig. 1 panels (C) and (F) and our previous observations of 18f infected FRhK-4 cells (Goswami et al., 2004; Kulka et al., 2009; Kulka et al., 2003), we hypothesized that the induction of cpe in clone 1 persistently infected FRhK-4, as well as A549, cells may involve rRNA cleavage, i.e., activation of the OAS/RNase L pathway. Therefore, we monitored rRNA cleavage in both FRhK-4 and A549 cells either uninfected or persistently infected with clone 1 at 37 °C as well as 33 °C (Fig. 2). While rRNA cleavage is not observed in uninfected FRhK-4 or A549 cells (Fig. 2, panels (A) and (B), respectively) at either temperature, both persistently infected cell lines exhibit a strong induction of rRNA cleavage at 4 and 6 days post-culture at 33 °C, but not at 37 °C (shown only for day 6 post-culture). Activation of RNase L in these cells appears restricted by both incubation temperature and prior virus infection. Interestingly, the activation of RNase L in both persistently infected cell lines occurs in conjunction with the development of cpe at 33 °C, as previously reported by Kulka et al. (2009) for the cytopathic strain HM175/18f in FRhK-4 cells at 37 °C. Unlike infection with 18f, however, the activation of RNase L in persistently infected cell lines does not occur at 37 °C.

Caspase activation in FRhK-4 or A549 cells persistently infected with HM175/clone 1

It has been previously shown that cpe/apoptosis in HM175/18f infected FRhK-4 cells is caspase-dependent (Goswami et al., 2004), therefore, we first sought to determine whether we could detect caspase activation in situ in both HM175/clone 1 persistently infected cell lines. Thus, both persistently infected cell lines were grown for 5 days at 33 °C or 37 °C prior to treatment with 10μM FITC-labeled z-VAD-fmk. This modified pan-caspase inhibitor binds to the active form of caspases at a concentration and treatment period insufficient to inhibit caspase activity (Goswami et al., 2004, data not shown). The results of fluorescent microscopy for these treated cells are shown in Fig. 3. Uninfected cells grown at 33 °C or persistently infected cells grown at 37 °C are negative for fluorescence while both infected cell lines are positive for fluorescence at 33 °C indicating that caspase(s) are activated in these cells. Fluorescent staining is characterized by a few cells with intense fluorescence and approximately 50% of the remaining cells exhibiting punctate, albeit weaker, fluorescence. We interpret the weak but uniform staining as consistent with the initiation of apoptosis and progressive development of cpe observed over time (4 to 6 days) in these cells at 33 °C. Indeed, the data suggests that RNase L activation (viz. rRNA cleavage) may precede the onset of apoptosis as previously observed for 18f infected cells (Goswami et al., 2004; Kulka et al., 2009).

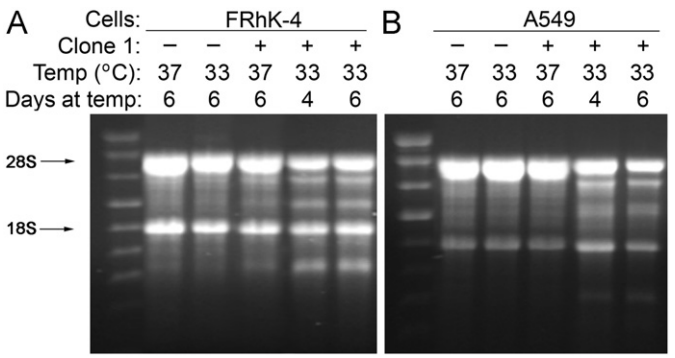

Fig. 2. Analysis of rRNA cleavage in FRhK-4 and A549 cells persistently infected with HM175/clone 1 and cultured at either 33 °C or 37 °C. Uninfected and clone 1 persistently infected FRhK-4 cells (A) and uninfected and clone 1 persistently infected A549 cells (B) were seeded at 4000 and 6000 cells/cm^2, respectively, cultured for 6 days at either 33 °C or 37 °C prior to harvest for cytoplasmic RNA, and analyzed (3.5 μg) for RNA cleavage by agarose gel electrophoresis (Goswami et al., 2004). The 28S and 18S rRNAs are identified with arrows. Cleavage of rRNA is not observed in uninfected cells or persistently infected cells at 37 °C.

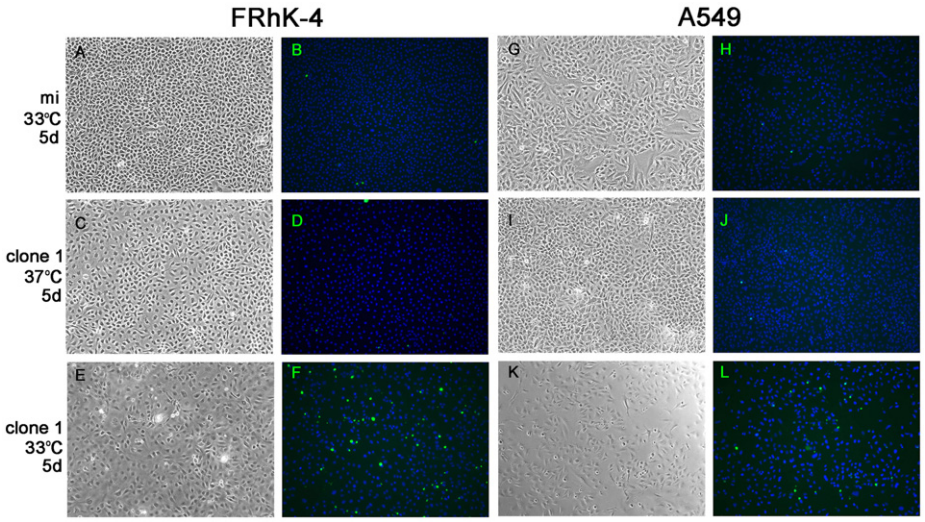

Fig. 3. Fluorescent microscopy for the examination of HM175/clone 1 persistently infected FRhK-4 and A549 cells treated with FITC-labeled z-VAD-fmk. Uninfected and clone 1 persistently infected FRhK-4 ((A)–(F)) and A549 ((G)–(L)) cells were seeded (in 5 cm^2 wells) at 4000 and 6000 cells/cm^2, respectively, then cultured for 5 days at either 33 °C ((A), (B), (E)–(H), (K), (L)) or 37 °C ((C), (D), (I), (J)). All cells were incubated in the presence of 10 μM FITC-labeled z-VAD-fmk for 4 h on day 5 in culture prior to cell fixation then subjected to brightfield ((A), (C), (E), (G), (I), (K)) and fluorescent ((B), (D), (F), (H), (J), (L)) microscopic examination as described in Materials and methods.

Cytopathic effect and apoptosis induced at 33 °C in HM175/clone 1 persistently infected cells are caspase-dependent

We next sought to determine whether apoptosis was actually occurring in persistently infected cells during incubation at 33 °C. To this end, activation of caspase-3 (viz. caspase-3 cleavage) and formation of DNA laddering were examined in these cells, as well as in uninfected cells as negative controls. Western blot analysis of VP-1 expression was used to monitor viral capsid expression as an indicator of virus replication concomitant with any development of apoptosis. The anti-VP1 antibody has been shown to detect HAV VP-1 protein when it is part of a larger precursor protein as well as in its fully processed form (Goswami et al., 2004; Kulka et al., 2009, 2003).

The results of the analyses of VP-1 expression, caspase-3 expression/cleavage and actin levels for uninfected and persistently infected FRhK-4 and A549 cells at 33 °C and 37 °C are shown in Fig. 4. Strong VP-1 expression (panel (A)) is detected in persistently infected cells at 33 °C and 37 °C indicating successful virus infection and replication in these cells. A non-specific cellular cross-reactive band is also present in uninfected FRhK-4 and A549 cells, though it migrates slightly higher than VP-1 upon close inspection. Despite the intensity of cpe observed in Fig. 1, expression of both partially processed (P1-2A and VP1-2A) and fully processed capsid protein VP-1 appear more robust at 33 °C, particularly in A549 cells, suggesting viral protein expression/processing is largely unaffected by those processes (viz. cell death) responsible for the cytopathic phenotype. Indeed, the increase in viral protein levels is commensurate with development of cpe in these cells. Most interesting, however, are the results indicating that the major effector caspase, caspase-3, is only cleaved/activated in infected cells at 33 °C and increases with the time of incubation (Fig. 4, panel (B)). This indicates that caspase pathway activation occurs in conjunction with development of cpe in both persistently infected cell lines. The levels of VP-1 and caspase-3 expression/processing are not due to differences in gel (lane) loading, as actin levels are essentially equivalent among all samples (Fig. 4, panel (C)).

To confirm whether apoptosis occurs in these infected cells, we obtained extracts derived from persistently infected FRhK-4 and A549 cells, as well as uninfected parental cells, incubated at either 37 °C or 33 °C for analysis of cytoplasmic DNA laddering.

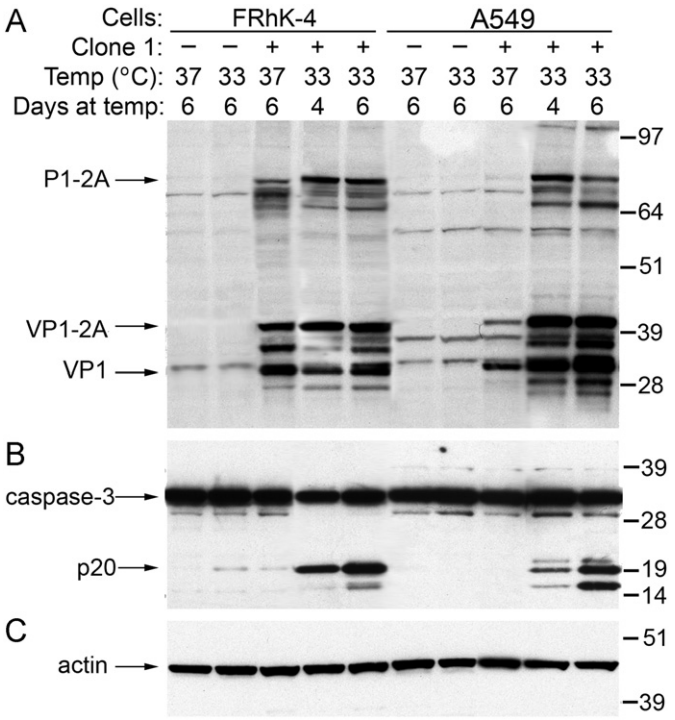

Fig. 4. The effect of temperature on the expression and processing of HAV viral capsid protein VP-1 and caspase-3 in clone 1 persistently infected FRhK-4 and A549 cells. Protein extracts (25 μg) were analyzed for VP-1 expression and processing in both uninfected and persistently infected cell lines (A). Cells were seeded at 4000 and 6000 cells/cm^2 respectively, and cultured for 6 days at either 33 °C or 37 °C prior to harvest for analysis by western blot using affinity purified anti-VP-1 antibody. The blots were stripped, washed and re-probed with anti-caspase-3 for analysis of caspase-3 expression and cleavage (B). The blots were re-stripped, washed and re-probed with anti-actin antibody (C). The positions of the HAV precursor proteins P1-2A and VP1-2A as well as the fully processed VP-1 are identified with arrows in panel (A). In panel (B), the positions of full-length caspase-3 (non-activated) and the p20 peptide of activated caspase-3 are identified with arrows. In some lanes, the mature p17 subunit is also visible.

The appearance of DNA laddering in the cytoplasm is an indicator of an activated apoptotic process (Brack et al., 1998; Goswami et al., 2004). Cleavage of DNA is also observed after 4 days at 33 °C

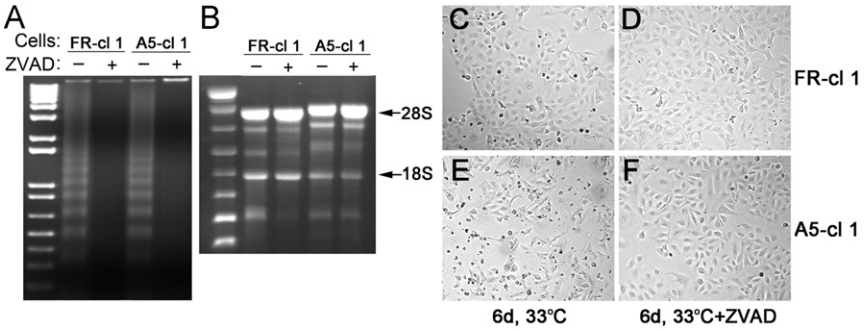

Fig. 5. The effect of z-VAD-fmk on ribosomal RNA cleavage, DNA laddering, and cytopathic effect in FRhK-4 and A549 cells persistently infected with HM175/clone 1 cultured at 33 °C. Total cytoplasmic nucleic acid extracts were obtained from persistently infected FRhK-4 (Fr-cl 1) and A549 (A5-cl 1) cells and analyzed for DNA laddering (A) and rRNA degradation (B) by agarose gel electrophoresis as previously described (Goswami et al., 2004). Cells were seeded at 4000 cells/cm^2 (Fr-cl 1) or 6000 cells/cm^2 (A5-cl 1) and cultured for 6 days at 33 °C in the absence (−) or presence (+) of 50 μM z-VAD-fmk (for the last 5 days in culture) prior to harvest for extraction of cytoplasmic nucleic acid. The 28S and 18S rRNAs are identified with arrows. Persistently infected FRhK-4 ((C) and (D)) and A549 ((E) and (F)) cells were seeded at 4000 and 6000 cells/cm^2 respectively, then cultured for 6 days at 33 °C in absence ((C) and (E)) or presence ((D) and (F)) of 50 μM z-VAD-fmk (for the last 5 days in culture). Cultures were observed over the 6-day incubation period by brightfield microscopy and images were obtained and shown for day 6 of the culture period.

in both persistently infected cell lines with stronger cytoplasmic DNA fragment staining observed in the infected FRhK-4 cell line (data not shown). As shown in Fig. 5 (panel (A)), apoptotic DNA laddering is evident in both FRhK-4 and A549 cells persistently infected with HM175/clone1 grown for 6 days at 33 °C. Neither persistently infected cells incubated at 37 °C nor uninfected cells incubated at either temperature show any evidence of DNA laddering (data not shown). The presence of DNA laddering is coincident with cleavage of caspase-3 shown in Fig. 4 (panel (B)) for the infected cell lines grown at 33 °C indicating a good correlation between these two events. Therefore, we examined the possibility of caspase-dependent apoptosis in both clone 1 infected cell lines by determining the effect of the pan-caspase inhibitor (z-VAD-fmk) on DNA laddering. As shown in Fig. 5 (panel (A)), incubation of either persistently infected cell line at 33 °C (for 6 days) in the presence of z-VAD-fmk (from days 1 to 6) completely abrogated DNA laddering. As previously reported for 18f infected FRhK-4 cells (Goswami et al., 2004), similar incubation and treatment of either persistently infected cell line with z-VAD-fmk had no effect on rRNA cleavage in either clone 1 infected cell line (Fig. 5, panel (B)) indicating that rRNA cleavage (and thus RNase L activation) is not a consequence of apoptosis in these cells. Importantly, we observed a concomitant and significant reduction of cpe ($>$ 90%) in infected FRhK-4 cells and A549 cells both treated with 50 μM z-VAD-fmk (Fig. 5, panels (D) and (F), respectively) compared to untreated, infected cells (Fig. 5, panels (C) and (E), respectively). Since z-VAD-fmk does not affect replication of HAV (Goswami et al., 2004; data not shown) at concentrations used in this study, the data indicate that HAV HM175/clone 1 permissive infection (persistent) is subject to temperature dependent induction of apoptosis resulting in the establishment of cpe in FRhK-4 and A549 cells.

The development of cpe, RNase L activity, and apoptosis are affected by time and temperature of incubation, and cell density seeding

During the course of our investigation we discovered that the onset, establishment, and maintenance of cpe, rRNA cleavage and DNA laddering were affected by a number of experimental variables, e.g., time of incubation at 33 °C, time of incubation (sequential) at 37 °C prior to (or following) incubation at 33 °C, and cell seeding ratio/density. For example, in persistently infected FRhK-4 cells (sub-cultured at ratios of 1:20–1:40) we observed that incubation at 37 °C for $\geq$ 4–5 days prior to incubating the cultures at 33 °C yielded cultures without evidence of cpe (data not shown). However, reducing the initial time at 37 °C to 1–2 days led to cpe (and DNA laddering) when the cultures were further incubated at 33 °C for at least 4–6 days (data not shown). Finally, lower sub-culture ratios (less than 1:20) negatively affected the onset of cpe and DNA (data not shown). We interpreted these results to suggest that cell density, particularly in relation to time of incubation at 33 °C, may influence whether induction of apoptosis (i.e., cpe) will occur.

In order to better control for the potential effect of cell density, we determined in preliminary experiments that seeding densities of approximately 4000 cells/cm^2 and 6000 cells/cm^2 for FRhK-4 and A549 cells, respectively, were adequate to yield cellular extracts of sufficient quantity for cytosolic nucleic acid analysis in combination with observation of cpe induction. Subsequent investigations were designed to examine several experimental parameters and representative results are shown in Table 1. For example, in both infected cell lines cultured at 33 °C (but not 37 °C) the development of cpe is observed at approximately

Table 1
Analysis of cpe, RNase L activity and apoptosis as a function of time and temperature of incubation and cell density in persistently infected cell lines.

Cell line[a]	Treatment[b]	Subculture Density[c]	Cpe[d]	rRNA Cleavage[e]	DNA Laddering[f]
FRhK-4/clone1	4d 33 °C	4,000	+	+	+
	6d 33 °C	4,000	+	+	+
	6d 37 °C	4,000	−	−	−
	6d 33 °C/1% FCS	4,000	+	+	+
	6d 37 °C/1% FCS	4,000	−	−	−
	6d 31.5 °C	4,000	+	+	+
	6d 33 °C	20,000	−	+	−
	6d 37 °C	20,000	−	−	−
A549/clone 1	4 d33 °C	6,000	±	+	±
	6d 33 °C	6,000	+	+	−
	6d 37 °C	6,000	−	−	−
	6d 33 °C	40,000	−	+	−
	6d 37 °C	40,000	−	−	−

[a] HM175/clone 1 persistently infected FRhK-4 (105–140 dpi) or A549 (70–141 dpi) cells. VP-1 expression was detected in all infected cells under all conditions of treatment[b] (data not shown). Uninfected FRhK-4 and A549 cells were also treated as infected cells, with all negative results (data not shown).

[b] Indicated cell lines were incubated at either 33 °C or 37 °C for indicated days in growth media containing 5% and 10% heat-inactivated FCS for FRhK-4 and A549 cells, respectively, unless otherwise indicated.

[c] Subculture density is given as number of cells per cm^2 culture vessel surface area.

[d,e,f] Results expressed as positive (+), weakly positive (±), or negative (−) for cpe, rRNA cleavage and DNA laddering as described in Materials and Methods.

4 days and the intensity of cpe and DNA laddering increase with time of incubation such that they remain positive at 6 days. Cleavage of rRNA is consistently observed under these incubation conditions. The destruction of the monolayer continues beyond 6 days and a concomitant increase in extent of rRNA cleavage and DNA laddering is likewise observed (data not shown). As shown in Table 1, neither a reduction in serum concentration to 1% nor a reduction in the incubation temperature to 31.5 °C had any significant inhibitory effect on the induction of cpe, rRNA cleavage or DNA laddering in persistently infected cells at 33 °C. In contrast, increasing the seeding density of persistently infected FRhK-4 cells to 20,000 cells/cm^2 completely abolishes the development of cpe even after 6 days at 33 °C. Interestingly, rRNA cleavage was not abolished with increased seeding density. Similar results are achieved with persistently infected A549 cells seeded at 6,000 cells/cm^2 versus 40,000 cells/cm^2 suggesting the results are not unique to a given cell type/species. In addition, we have consistently observed throughout our investigation that cpe, rRNA cleavage and DNA laddering are not observed in either persistently infected cell lines incubated at 37 °C (Table 1) or in either parental uninfected cell lines incubated at 33 °C or 37 °C (data not shown).

In summary, these results indicate that in either persistently infected cell line a cytopathic effect is accompanied by the detection of rRNA cleavage (i.e., RNase L activation) and caspase-dependent DNA laddering (i.e., apoptosis) at 33 °C. While the presence of DNA laddering in the absence of rRNA cleavage or cpe has not been observed, rRNA cleavage occurs in the absence of cpe and DNA laddering when cells are seeded at a higher density sufficient to achieve confluence in less than 3–4 days at 33 °C.

Cell density dependence of cpe induction in A549 cells acutely infected with HM175/clone 1

We considered that the induction of cpe could be due to the effect of putative mechanism(s) and/or factor(s) responsible for maintenance of a persistent state of infection. As a consequence, cpe may not occur during an acute HAV infection with HM175/clone 1. To explore this possibility, we acutely infected confluent A549 cells with HM175/clone 1 from the ATCC stock vial used to establish our persistent lines at 37 °C. As shown in Fig. 6 (panels (A) and (B)) at 10 dpi, cpe does not occur at either 33 °C or 37 °C when cells were infected at confluency. However, given that subculture seeding density appears to affect the onset of cpe in persistently infected cells (Table 1), we sought to determine whether cpe occurs when sub-confluent A549 cells were used for infection. Indeed, a cytopathic effect is readily observed at 33 °C, but not 37 °C, at 10 dpi following sub-confluent infection with HM175/clone 1 (Fig. 6, panels (D) and (C), respectively; Table 2) with onset initially observed at 6 days pi (data not shown). A cytopathic effect was also observed following acute infection with HM175/clone 1 in FRhK-4 cells (Table 2) whereby cpe (albeit weaker) is observed only at 33 °C and when cells were infected at subconfluency. These results indicate that induction of apoptosis/cpe at 33 °C does not require establishment of a persistent infection.

Acute infection with either HAV strain HAS-15 or LSH/S and induction of cpe in FRhK-4 and A549 cells as a function of incubation temperature and cell density

To further examine whether the temperature/cell density-dependent cpe phenotype may be unique to the HM175/clone 1 strain of HAV, we obtained two additional non-cytopathic HAV (culture-adapted) strains, LSH/S and HAS-15, from a commercial source for investigation of cpe at 33 °C following acute infection of either FrHK-4 or A549 cells. As indicated in Table 2, all three

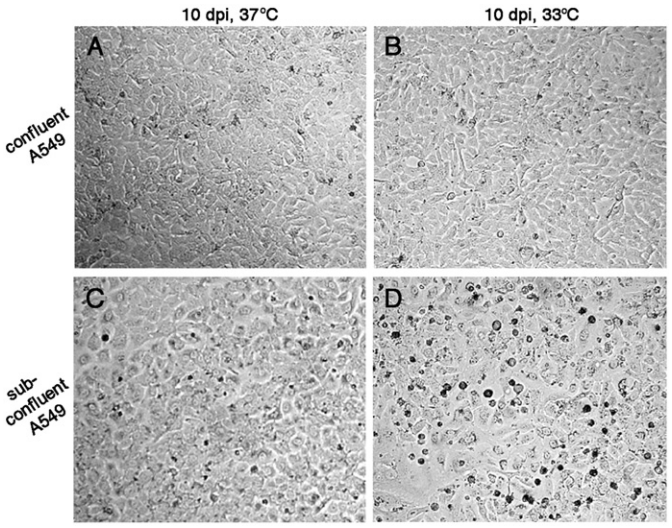

Fig. 6. Brightfield microscopy for the examination of the effect of cell density on induction of cpe at 33 °C and 37 °C in HM175/clone 1 infected A549 cells. A549 cells were seeded at 12,000 ((A) and (B)) and 600 ((C) and (D)) cells/cm^2, cultured for 4 days at 37 °C prior to infection with HM175/clone 1 at an moi of 5–8, and infected cell cultures were incubated at either 37 °C ((A) and (C)) or 33 °C ((B) and (D)). Cultures were observed over a 10-day incubation period by brightfield microscopy and images were obtained and shown for day 10 of the culture period.

strains exhibit cpe following acute infection of sub-confluent cells and incubation at 33 °C, but not at 37 °C. In contrast, there was no evidence of cpe following infection of confluent cells with any of the strains regardless of the temperature of incubation. Interestingly, as with HM175/clone 1, there was variability in cpe depending on the virus strain/cell line combination. For example, while onset of cpe occurs at 33 °C in FRhK-4 and A549 cells infected with HAS-15, the cpe is relatively more intense in FRhK-4 cells. The results with LSH/S are essentially the reverse of those obtained with HAS-15 whereby strong cpe was observed in A549 cells with little or no cpe detected in similarly infected FRhK-4 cells. These differences are not readily explained by an absence of infection since strong expression of VP-1 was detected in all sub-confluent infected cells cultured at 33 °C (data not shown). In summary, the results indicate that the induction of cpe following acute infection with three different HAV strains does occur, but does so as a function of temperature, cell density at the time of infection, and virus strain/cell line combination for infection.

Analysis of RNase L activation and apoptosis following sub-culture of cells acutely infected with HM175/clone 1, LSH/S or HAS-15

Interestingly, previous investigations into the establishment of persistently infected lines have included reduced temperature culture conditions (i.e., <37 °C), yet there has been no report of an induction of cpe under these conditions. We believe the absence of cpe in those studies was at least in part due to the use of confluent cells to establish a persistent infection (Anderson, 1987; Dotzauer et al., 1994; Goswami et al., 2004; Kulka et al., 2009; Simmonds et al., 1985; Vallbracht et al., 1984) as we have confirmed the absence of cpe at 33 °C following the acute infection of FRhK-4 or A549 cells at confluency with three different virus strains, HM175/clone 1, HAS-15 or LSH/S (Fig. 6 and Table 2). However, we were curious to determine whether the apparent density-dependent restriction/inhibition of cpe in infected cells was irreversible. To this end, acutely infected (confluent) cells were cultured at 37 °C or 33 °C for 3 dpi before the cultures were sub-cultured (at low density) for continued incubation (5 days) at the respective temperatures of 37 °C or

Table 2
Analysis of cpe as a function of time and temperature of incubation and cell density in acutely infected cells.

cpe[a]				cpe[a]			
Cell line/virus	Density[b]	33 °C	37 °C	Cell line/virus	Density[b]	33 °C	37 °C
FRhK-4/clone1	Confluent	–	–	A549/clone1	Confluent	–	–
	Sub-confluent	+	–		Sub-confluent	++	–
FRhK-4/HAS-15	Confluent	–	–	A549/HAS-15	Confluent	–	–
	Sub-confluent	++	–		Sub-confluent	+	–
FRhK-4/LSH/S	Confluent	–	–	A549/LSH/S	Confluent	–	–
	Sub-confluent	±	–		Sub-confluent	+++	–

[a] Infected cell cultures were incubated at either 33 °C or 37 °C for determination of cpe at 10 dpi. Results expressed as a weakly positive (+), positive (++), strongly positive (+++), or negative (–).

[b] To obtain either confluent or sub-confluent cell monolayers at the time of virus infection, FRhK-4 or A549 cells seeded at 400 and 600 cells/cm^2, respectively (for sub-confluency), or 10,000 and 12,000 cells/cm^2, respectively (for confluency), and cultured for 4 days at 37 °C prior to infection with the indicated virus at an moi of 5.

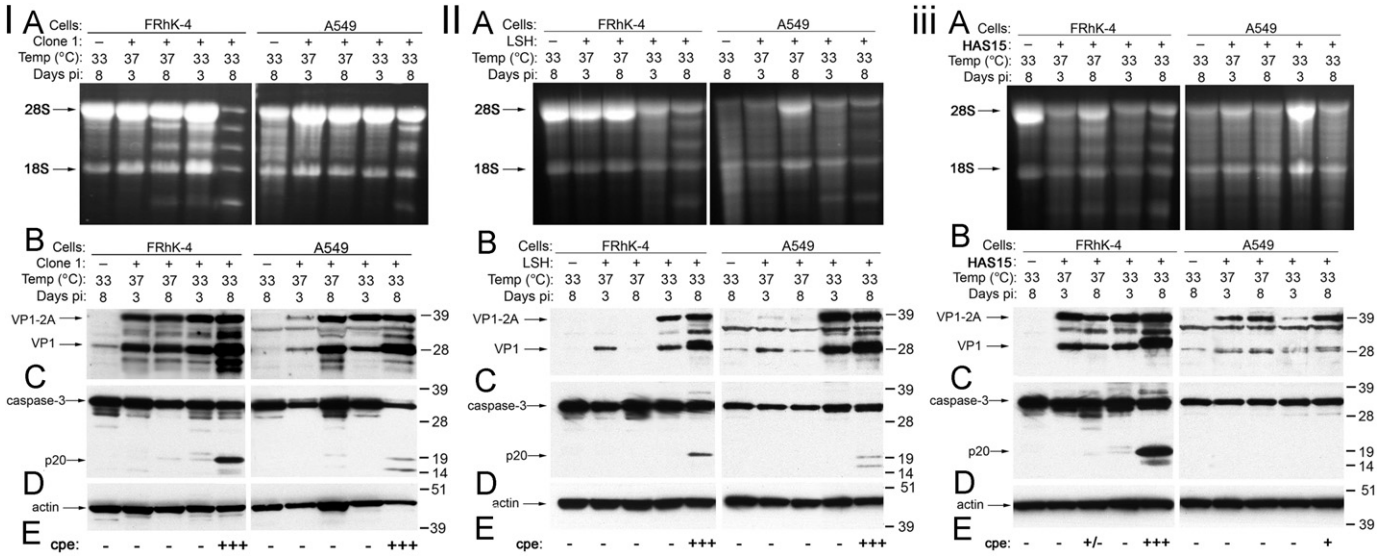

Fig. 7. The effect of subculture and temperature on rRNA cleavage, the expression and processing of HAV viral capsid protein VP-1, caspase-3, and the induction of cpe in FRhK-4 or A549 cells infected with different HAV strains. Confluent FRhK-4 or A549 cells were either mock (I–III), HM175/clone 1(I), LSH/S (II), or HAS-15 (III) infected (moi. of 5–8) and cultured at either 33 °C or 37 °C for 3 days. At 3 dpi, the cells were subcultured at low density (1:20 and 1:30 for mock or infected, FRhK-4 and A549 cells, respectively), and incubation continued at the same temperature for an additional 5 days (i.e., 8 dpi total). Cultures were harvested as replicate cell pellets to obtain cytoplasmic RNA obtained for analysis (3.5 μg) of RNA cleavage by agarose gel electrophoresis (A) (Goswami et al., 2004), and protein extracts for immunoblot analysis (25 μg) of HAV VP-1 expression and processing (B). The blots were stripped, washed and re-probed with anti-caspase-3 for analysis of caspase-3 expression and cleavage (C). The blots were re-stripped, washed and re-probed with anti-actin antibody (D). The 28S and 18S rRNAs are identified with arrows in panel (A). The positions of the HAV precursor protein VP1-2A as well as the fully processed VP-1 are identified with arrows in panel (B). In panel (C), the positions of full length caspase-3 (non-activated) and the p20 peptide (cleavage product) are identified with arrows. In some lanes, the mature p17 subunit is also visible.

33 °C (8 dpi total). Results obtained using all three HAV strains in both cell lines are shown in Fig. 7. The development of cpe (sections I–III, panel (E)) was not observed in any infected cells when incubated at 37 °C for either 3 dpi or following subculture growth to 8 dpi with the exception of scattered cpe-like "foci" in HAS-15 infected FRhK-4 cells at 8 dpi (scored as ± in Fig. 7, III, panel (E)). In contrast, however, a strong cytopathic effect was observed in both cell lines at 33 °C at 8 dpi, but not 3 dpi, regardless of the virus strain. The broadest cpe was observed in FRhK-4 cells infected whereby a cytopathic effect is induced following infection with each of the three strains at 33 °C and 8 dpi (Fig. 7 I–III, panel (E)). In A549 infected cells there was variation in the cpe with the strongest cpe following infection with either LSH/S or HM175/clone 1, and the weakest following HAS-15 infection. Caspase-3 activation (i.e., cleavage) was only detected in infected cells under incubation conditions that facilitated induction of strong cpe. The weak cpe observed at 8 dpi at 33 °C in HAS-15 infected A549 cells may be due to non-apoptotic events. It is not due to an insufficient time pi to permit further development of cpe and/or apoptosis, as these cultures were grown with routine weekly subculturing for two more weeks at 33 °C with no sign of cpe development, rRNA cleavage, or caspase-3 activation (data not shown). Interestingly, cpe/apoptosis does not occur without rRNA cleavage as we have also shown for persistently infected cells (Figs. 2, 4, 5 and Table 1). However, rRNA cleavage can occur in the absence of cpe/apoptosis as is shown for HM175/clone 1 infected FRhK-4 cells at 8 dpi/37 °C and 3 dpi/33 °C (Fig. 7 I, comparing panels (A), (C), and (E)), and HAS-15 infected FRhK-4 cells likewise at 8 dpi/37 °C and 3 dpi/33 °C (Fig. 7 III, comparing panels (A), (C), and (E)). Since the infection/culture protocol used to generate the results in Fig. 7 may be considered passage one of a persistently infected cell line, it is interesting to note similar results are obtained in longer passaged, persistently infected cells (Figs. 2 and 4 and Table 1).

Immunoblot analysis was also used to detect viral protein VP-1 expression as an indicator of virus replication and the results are shown in Fig. 7 groups I–III, panel (B). With the exception of LSH/S infected FRhK-4 or A549 cells where levels of VP-1 decrease from 3 dpi to nearly (or completely) undetectable levels at 8 dpi and 37 °C, both cell lines supported the replication of all three

viruses as evidenced by the detection of partially [P1-2A (not shown) and VP1-2A proteins] and fully processed VP-1. The restricted replication at 37 °C is not unexpected given the reported predilection for replication of LSH/S at 32 °C (Fineschi et al., 1991; Pellegrini et al., 1993). Importantly, viral capsid protein expression, and therefore viral gene replication, appears to occur in cells undergoing cpe/apoptosis. Interestingly, save for the results in HAS-15 infected A549 cells, the levels of VP-1 (particularly the fully processed protein) are greater at 33 °C than at 37 °C, with the greatest difference observed at 8 dpi. Generally, the levels of actin detected by immunoblot were similar within experimental group samples with the exception of HM175/clone 1 infected A549 cells at 8 dpi 37 °C and 33 °C (Fig. 7 group I, panel (D)). These samples reveal a 3-fold increase and nearly 0.7-fold decrease (by densitometric analysis, data not shown), respectively, compared to other samples in the series and likely reflects gel loading differences. However, these results do not affect the interpretation of the results regarding VP-1 expression, cpe induction and caspase-3 activation. In summary, these results suggest: (i) the induction of cpe/apoptosis is not irreversibly blocked by prior (acute) infection in confluent cells and does not adversely affect viral gene expression/replication; (ii) the reduction of incubation temperature may potentiate viral gene expression; and (iii) there is a positive correlation between activation of caspase-3 and induction of cpe in these infected cells suggesting apoptosis is responsible for the observed cytopathogenicity as we demonstrated for persistently infected cell lines (Fig. 5).

In order to confirm whether viral gene replication is occurring in infected cells, a portion of the cytoplasmic RNA obtained for the analysis of rRNA cleavage was held in reserve and used for negative strand RT-PCR to determine the presence of viral negative strand synthesis (Kulka et al., 2009). Amplified samples representing infected cells at 8 dpi were analyzed by agarose gel electrophoresis. Negative strand synthesis, and therefore viral replication, is detected at 8 dpi in infected cell extracts at either 33 °C or 37 °C, except in LSH/S infected FRhK-4 and A549 cells at 37 °C where the HAV amplicon was not detected and weakly detected, respectively (data not shown). The latter results suggest that a reduction in virus replication may be responsible for the reduction in VP-1 expression observed in these cells (Fig. 7 group II, panel (B)). Additionally, the presence of negative strand synthesis in all infected cells undergoing cpe confirms that induction of cpe/apoptosis does not block virus replication. The results also indicate that our primer set is suitable for the detection of all three strains of HAV and may be used to develop an RT-qPCR protocol for determining whether virus replication/genome synthesis rates differ among the infected cells.

Discussion

HAV replication is protracted, its growth is cell-type restricted, and it is capable of establishing a persistent infection in vitro that is particularly evident following continuous passage (subculture) of an infected cell line (Hollinger, 2001; Nainan et al., 2006; Ross et al., 1991). Serial passage of wild-type virus strains, derived from culture supernatants or cell extracts, can result in the generation of cell-culture adapted strains that typically replicate faster and express viral proteins earlier post-infection than the parental wild-type, but remain non-cytopathic. The serial passage of cell-culture adapted HAV strains results in the emergence of even faster growing strains capable of inducing a cytopathic effect (cpe) during virus replication in cell lines such as FRhK-4 and B-SC-1 (Anderson, 1987; Brack et al., 1998; Cromeans et al., 1989; Cromeans et al., 1987; Lemon et al., 1991; Nasser and Metcalf, 1987; Venuti et al., 1985).

In contrast to those studies, our current investigation reveals that culture-adapted non-cytopathic strains may already possess a cytopathic phenotype that is induced under appropriate incubation conditions. Working initially with persistently HM175/clone 1 infected FRhK-4 and A549 cell lines, we determined that reducing the temperature ($\leq$ 34 °C) of incubation during subculture passage of these lines leads to the development of an easily visible cytopathic effect that appears co-dependent on the subculture seeding density. The intensity and severity of cpe increases with the time of incubation at 33 °C whether or not there was a period of prior incubation at 37 °C. We have observed that persistently infected cell cultures seeded at densities whereby they reached confluency at or before 3–4 days at $\leq$ 34 °C gave acceptable cell quantities for analyses (immunoblot and cytosolic nucleic acid), but appeared less likely to develop any remarkable cpe. Ultimately, we determined that seeding densities of 4000 and 6000 per cm^2 of culture area for FRhK-4 and A549 cells, respectively, were sufficient to permit multiple comparative analyses while at the same time yielding a cytopathic effect. At no time in our investigation did we observe the induction of phenotypic changes in uninfected FRhK-4 or A549 cells similar to those observed in either persistently or acutely infected cells at $\leq$ 34 °C.

We considered it a possibility that the apparent temperature/cell density-dependent cpe phenotype in persistently infected cells may have been the result of either a viral or cellular adaptive process that occurred during continuous passage of persistently infected cells, or a property unique to the HM175/clone 1 strain of HAV. The former would be surprising given our usage of two different cell lines (monkey kidney versus human lung) to establish the persistent infections. However, our study also included investigation of the effect of temperature and density following acute infection in FRhK-4 and A549 cells using HM175/clone 1 as well as two additional (and genotypically different (Robertson et al., 1992) and M. Mammel, personal communication)) HAV strains. The results indicate that induction of cpe can be made to occur in acutely infected cell lines with three different virus strains and was restricted by similar conditions of incubation temperature and cell density (Fig. 7 and Table 2). One can conclude, therefore, that induction of apoptosis/cpe at 33 °C does not require establishment of a persistent infection and is not a property unique to the HM175 strain of HAV. Alternatively, we considered the possibility that mechanism(s) responsible for cpe in persistently infected cells may be different from those occurring in acutely infected cells, such that in persistently infected cells a selection and/or enrichment of a cytopathic mutant may be occurring as a consequence of continuous subculture of persistently infected cell lines. This seems an intriguing possibility particularly since investigators have used HM175 persistently infected cells as a part of the overall subculture scheme for generating cytopathic mutants (Anderson, 1987; Cromeans et al., 1987; Lemon et al., 1991). In their studies, however, obtaining the cpe mutant usually required clonal isolation following acute serial passage(s) in order to obtain the mutant and fully demonstrate cpe in infected cells, and the process of clonal isolation and selection (e.g., via limiting dilution, foci or plaque formation) uses acute infection or "serial" passage depending on the number of cloning steps. In contrast, Anderson (1987) reported cytopathology without the need for clonal isolation of the virus (derived from persistently infected cells) but he did employ an acute subculture passage prior to observing the cytopathic effect. Despite their results, we do not believe we have enriched and/or selected for a cytopathic mutant of HM175 under conditions of persistent cell passage since (i) neither clonal isolation nor subculture passage was necessary to establish cpe, (ii) the cytopathic effect occurs in persistently infected cells at

33 °C both at early (8 dpi and 60 dpi) and late passage (> 200 dpi) despite culture maintenance at 37 °C — a temperature not conditional for the cpe phenotype, (iii) a single acute infection with HM175/clone 1 was used to establish persistently infected cell lines of two different (human, A549 and monkey, FRhK-4) cell origins without prior serial passage of the virus (in our laboratory), and (iv) the development of cpe also occurs in acutely infected FRhK-4 and A549 cells (Figs. 6 and 7, or Table 2) without serial virus passage (in our laboratory) and using the same virus stock as that used to establish the persistently infected cell lines.

Our results obtained following acute infection with the three different strains of HAV similarly invoke two alternative explanations for the temperature and cell density co-dependent onset of cpe. One involves an assumption that a sub-population of cytopathic conditional mutants arises spontaneously following acute infection and growth of the non-cytopathic virus, and the other explanation assumes that a cytopathic conditional mutant sub-population already exists within the non-cytopathic strain population (commercial stock) that was generated during serial passage of the stock. Consistent with the pre-existent mutant sub-population theory, we have observed cellular destruction initially occurring in only a portion of the cell monolayer and such a limited destruction may be interpreted as the induction of cpe following infection by a mutant subset of viruses. The existence of a putative conditional mutant within multiple commercial virus strains could have occurred as a consequence of serial passage of virus (or persistently infected cells) that typically occurs during continuous large scale virus production (Ali, 2002; Beneduce et al., 1995; Brack et al., 1998; Cromeans et al., 1989; Divizia et al., 1986; Lemon et al., 1991; Nasser and Metcalf, 1987; Venuti et al., 1985). However, we believe this scenario improbable since virus production would have to generate the same conditional mutation(s) for three different HAV strains. Rather, we believe the observed temporal onset and progression of cpe to be a consequence of the asynchronous replication of HAV. Indeed, this has been proposed as the reason why the onset of demonstrable viral antigen, production of infectious virus, etc are not similarly observed in, nor achieves a maximum level, in all cells at the same time, particularly at moi ≥ 5 whereby all cells have been shown to be infected (Anderson et al., 1988; Cho and Ehrenfeld, 1991; De Chastonay and Siegl, 1987; Funkhouser et al., 1999). Regarding the argument for spontaneous mutation during acute infection, we question the probability of mutations spontaneously arising in two different genotype strains of HAV [IA for HAS-15 and IB for HM175 and LSH/S] (Robertson et al., 1992 and Mr. M. Mammel, personal communication) that would ultimately give rise to nearly identical mutants expressing the same conditional, cytopathic phenotype. Also, the generation of a putative mutant under selective/permissive conditions in the absence of serial virus passage seems unlikely since it would had to have occurred during both the 10-day acute infection (Fig. 6 and Table 2), as well as the acute infection coupled with subsequent subculture (Fig. 7), which have short incubation periods. Indeed, we must again consider the alternative possibility that culture-adapted non-cytopathic strains may already possess a cytopathic phenotype that is induced under relevant incubation conditions such as decreased cell density and reduced incubation temperature.

With regard to the relevance of cell density and incubation temperature to cpe, the results from acute infection with and without subsequent subculture of the infected cells (Table 2 versus Fig. 7) are interesting. For example, persistent HM175/clone 1 infection of either cell line seeded at low density and 33 °C gave similar positive results regarding induction of cpe (Figs. 1, 3 and 5; Table 1). When either persistently infected cell line was seeded at a high density, there was no induction of cpe in either cell line despite incubation at 33 °C (Table 1). The absence of cpe at 33 °C following acute infection with HM175/clone 1 (10 dpi) at confluency (Table 2) was reversed in both cells when these infected cells were subcultured (at 3 dpi) for an additional 5 dpi (8 dpi and 33 °C; Fig. 7 I, panel (E)). A similar reversal was also observed for both subcultured lines acutely infected at confluency (33 °C) with LSH/S, and subcultured FRhK-4 cells infected at confluency with HAS-15 (compare Table 2 and Fig. 7 II and III, panel (E)). These results indicate that, with the exception of HAS-15 infected A549 cells, the absence of cpe observed in cells acutely infected at confluency is not necessarily irreversible provided subsequent incubation is at the permissive temperature. Persistently infected cell cultures maintained and sub-cultured at confluency at 37 °C, also demonstrate cpe following their subculture at 33 °C (Figs. 1–5, Table 1) suggesting the absence of cpe in cells infected at confluency and incubated at 37 °C (Table 2) is likely reversible. It is interesting that acute infection at low cell density with some virus-cell combinations directly resulted in a cytopathic effect at 33 °C (10 dpi) without a need for subculture (Table 2). It is therefore tempting to speculate that it may not be the act of subculture, but rather low density seeding at the time of or during infection at 33 °C that is important for induction of cpe. Ultimately, neither of the permissive culture parameters (33 °C and cell density) appears individually capable of or responsible for induction of cpe. Hence, both parameters may be equally critical and necessary for development of a cytopathic effect and therefore may be considered co-dependent factors, thus explaining why several investigators that have used temperatures < 34 °C during acute infection/serial passage of HAV or subculture of persistently HAV-infected cells have not observed a cytopathic effect (De Chastonay and Siegl, 1987; Fineschi et al., 1991; Frösner et al., 1979; Pellegrini et al., 1993; Provost et al., 1982; Siegl et al., 1984a, 1984b).

We have shown that the cytopathic effect is primarily due to caspase-dependent apoptosis since treatment with z-VAD-fmk abrogates both the development of cpe and DNA laddering in both infected cell lines. Importantly, it appears that cpe/apoptosis does not block virus replication/gene expression as indicated by the increase in levels of VP-1 concomitant with increasing cpe, activation of caspase-3 and clearly observable DNA laddering. Further investigations may include analysis of negative strand synthesis (as was done for acutely infected cells) or titration of infectious virus produced in these cells in order to confirm ongoing virus replication during drug treatment. We have observed "residual" cpe in persistently infected, z-VAD-fmk treated cells (approximately 1% in FRhK-4/clone 1 and 5% in A549/clone 1 cell lines, respectively) and conclude it is likely a result of the cumulative and detrimental effects on the overall health of the host cell(s) due to continuing virus replication and robust expression of viral proteins in the presence of the drug, or the effects of activated RNase L. We can conclude from our results that the induction of cytopathogenicity and apoptosis/cpe occurs only in the presence of virus gene expression or replication. On the other hand, the reverse does not appear to be so, suggesting that virus replication and/or gene expression are required, but not the sole factor, in affecting whether cpe/apoptosis will occur in virus infected cells. It is interesting to note, however, that the levels of VP1 expression and processing are generally higher in acutely infected cells at 33 °C than 37 °C for all three viruses (Fig. 7 I–III, panel (B)) in both lines, save HAS-15 infected A549 cells. A similar effect of reduced temperature is also observed for HM175/clone 1 persistently infected A549 cells. Thus, increased viral gene expression, and possibly replication, correlated with caspase-3 activation and apoptosis may be factors in the induction of cpe at 33 °C.

We made an initial investigation into defining what temporally related process(es) may be functioning to permit induction of a cytopathic effect following infection with non-cytopathic

strains of HAV. We sought to determine whether rRNA cleavage (a consequence of RNase L activation (Goswami et al., 2004; Kulka et al., 2009, 2003; Nilsen et al., 1982; Silverman et al., 1983; Wreschner et al., 1981)) is occurring prior to induction of cpe/apoptosis in HM175/clone 1, HAS-15 and LSH/S infected cells as was previously reported for FRhK-4 cells infected with the cytopathic HAV strain 18f (Kulka et al., 2009). Since the pattern of rRNA cleavage (Figs. 2, 5, and 7) is indicative of an activated RNase L pathway, we believe that the 2′-5′oligoadenylate synthetase (2-5OAS)/RNase L pathway has been activated in these cells as a result of viral dsRNA structures generated during replication and/or viral RNA folding (Bisbal and Silverman, 2007; Player and Torrence, 1998). These dsRNA structures function as activators of the OAS family of proteins that subsequently synthesize unique 2–5 A intermediates and ultimately activate the latent, endogenous RNase L. While HAV and coxsackievirus have been shown to be refractory to this canonical antiviral pathway (Kulka et al., 2009), the activation of RNase L has also been implicated in several cellular functions including in the induction of apoptosis (Castelli et al., 1998; Rusch et al., 2000; Zhou et al., 1998), although the mechanisms by which these two processes are connected are not well understood.

In established, persistently HM175/clone 1 infected FRhK-4 or A549 cells there was little or no detection of rRNA cleavage at 37 °C (Fig. 2, Table 1; Kulka et al., 2009), yet strong rRNA cleavage was observed in these cells when at an incubation temperature of 33 °C (4–6 dpi) whether seeded at high or low density (Fig. 2, Table 1). The cleavage of rRNA was not examined following continuous acute infection at 10 dpi (Table 2). However, at 3 dpi rRNA cleavage was not observed in any of the acutely infected A549 cells at either 33 °C or 37 °C, while cleavage was observed in 3 days post-infected FRhK-4 cells at 33 °C and weakly following HAS-15 infection at 37 °C Fig. 7 I–III, panel (A)). The passage (albeit first) of these "persistently" infected cells at 33 °C resulted in the RNase L activation in all except HAS-15 infected A549 cells and RNase L activation at 37 °C in HM175/clone 1 or HAS-15 infected FRhK-4 cells (Fig. 7). These results suggest that 33 °C favors RNase L activation in acutely infected FRhK-4 cells while subculture and growth at either temperature or 33 °C alone may remove this restriction depending on the cell line. The absence of cpe (and possibly RNase L activation) in LSH/S infected FRhK-4 cells at 37 °C (Table 2 and Fig. 7 II, panel (E)) is likely due a lack of sustained growth and replication growth as measured by loss of negative strand synthesis and VP-1 expression (data not shown and Fig. 7 II, panel (B)). The reason for the absence of cpe and RNase L activation in HAS-15 infected A549 cells, despite virus growth under conditions permissive for cpe in FRhK-4 cells and viral gene expression (albeit weaker than in FRhK-4 cells), is currently unknown. However, this outcome is consistent with the essentially negative cpe observed for HAS-15 acutely infected confluent A549 cells at 33 °C and 10 dpi (Table 2). We do not currently know whether differences exist among the infected cell lines with respect to viral replication rates. Such differences may affect the quantities of dsRNA sufficient and available to activate OAS that could account for the differential activation of RNase L observed among the infected cells under conditions permissive (or non-permissive) for induction of cpe. While a putative role for RNase L activation in the induction of the cpe phenotype is intriguing, its actual contribution remains uncertain in the absence of further investigation of virus replication rates or analysis of RNase L activity following virus infection and growth in permissive cell lines lacking OAS or RNase L. If the activation of RNase L is involved in the overall process of cpe induction, then the data appear to suggest the possibility of a two-step process requiring the activation of RNase L as a prerequisite to induction of apoptosis/cpe.

While previous investigations describe persistent cell culturing and acute infection (with and without further serial passage of the virus) at lower temperatures, to the best of our knowledge they have not used the virus strain/cell line combinations and/or sub-culturing strategies following initial acute infection that are described in our investigation (Anderson, 1987; Cromeans et al., 1987; De Chastonay and Siegl, 1987; Fineschi et al., 1991; Frösner et al., 1979; Lemon et al., 1991; Pellegrini et al., 1993; Provost et al., 1982; Siegl et al., 1984a, 1984b). Since we have proven that the induction of cpe is not restricted to a single cell type, yet have observed that cpe does not occur in all cell types with the same virus strain, we believe there is an important cellular contribution to the cpe effect. To this end, we briefly considered the involvement of cellular "cold shock" proteins, but preliminary data was inconclusive. It is tempting to entertain the possibility of the involvement of cell cycle-regulatory proteins which could help explain the low density requirement. Taken *in toto*, expression of a cpe phenotype by a non-cytopathic HAV is the result of multi-step process that appears co-dependent on incubation temperature and cell density as well as the activation of relevant cellular processes by a viral component requiring virus replication. These culture parameters may be responsible for affecting molecular processes such as virus replication and/or translation rates, or the presence/absence of critical cell regulatory factors (in part determined by the cell species) that ultimately may regulate the activation of the RNase L pathway and downstream events such as cpe/apoptosis. Ultimately, it would be both interesting and important to determine whether the induction of the cpe phenotype may be possible following cell culture infection with wild-type strains under the described culture conditions. Any attempt to investigate the induction of cpe with wild-type strains may also require co-investigation of the viral and cellular factors necessary to enhance virus replication. In this regard, understanding the mechanism(s) by which the non-cpe strains exhibit a cpe phenotype under the restricted conditions of reduced incubation temperature and cell density may provide the information necessary to reveal the molecular events that may regulate the non-cpe phenotype of wild-type strains. It is our belief that these and future investigations may ultimately lead to the development of methodologies (e.g., plaque assay) that can quantitatively measure the production of infectious virus.

Materials and methods

Cells and viruses

The HM175/clone 1, HAS-15 and LSH/S strains of HAV were obtained from ATCC (Manassas, VA). These non-cytopathic cell culture-adapted strains were originally derived from human isolates (i.e., wild-type strains) (Bradley et al., 1984; Daemer et al., 1981; Fineschi et al., 1991). In our laboratory the FRhK-4 (fetal rhesus monkey kidney) cell line was originally obtained from Dr. G. Kaplan (Center for Biologics Evaluation and Research, FDA) and cultured in MEM supplemented with pyruvate and non-essential amino acids (Invitrogen, Gibco Cell Products, Carlsbad, CA) containing 5% heat-inactivated fetal bovine serum. The A549 (human lung carcinoma) cell line purchased from ATCC was cultured in F-12K medium (ATCC) except with 10% heat-inactivated fetal bovine serum. Procedures for the establishment and routine maintenance of HM175/clone 1 persistently infected A549 cell cultures were as previously described for the establishment of HM175/clone 1 persistently infected FRhK-4 cells (Kulka et al., 2003; 2009; Goswami et al., 2004). Persistently infected cells were continuously sub-cultured at a split ratio (typically 1:30 to 1:40) sufficient to yield confluent cultures in 6–7 days and

grown at 37 °C and were the source of persistently infected cells used throughout the course of this investigation. These persistently infected cells ranged from 70–180 days post-infection for use in experimental analysis.

Antibodies

Rabbit anti-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibody to caspase-3 was purchased from Cell Signaling (Danvers, MA). Polyclonal rabbit antisera to HAV capsid protein VP1 has been previously described by Kulka et al. (2009). Goat anti-rabbit and goat anti-mouse horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from Pierce Biotechnology Inc. (Rockford, IL).

Virus infection and caspase inhibitor treatment

For acute infections, confluent FRhK-4 or A549 cells were mock, HM175/clone 1, HAS-15 or LSH/S infected at an moi of 5–8 (90 min adsorption at 37 °C followed by removal of adsorption) for the indicated times at 33 °C or 37 °C in growth media containing either 1% (FRhK-4) or 10% (A549) heat-inactivated FBS. Sub-confluent cultures were obtained by seeding at 400 (FRhK-4) or 600 (A549) cells per cm^2 and culture incubation was at the indicated temperatures for 4 days prior to infection. Replicate cultures were similarly seeded and incubated to provide average cell counts prior to virus (or mock) infection at an moi of 5. Infected (or mock) cell cultures were either maintained at their respective temperatures or were harvested by scraping. Washed (once with cold PBS) cell pellets were obtained for subsequent nucleic acid or protein extraction (Kulka et al., 2003; Goswami et al., 2004). Persistently HM175/clone 1 infected FRhK-4 and A549 cells were seeded at the indicated cell densities or subculture split ratios, and cultured in growth media at either 37 °C or 33 °C for indicated times, and under the given conditions to obtain cell pellets for nucleic acid or protein extraction as described above for acutely infected cells. During the course of these studies and prior to obtaining cells pellets, cultures were routinely observed and digital images taken using light microscopy. We have observed that development of cpe was independent of the culture vessel size.

For treatment with pan-caspase [z-VAD-fmk (Promega Corp., Madison, WI)] inhibitor, persistently infected cell lines were seeded and cultured for 6 days at 33 °C in normal growth media. Growth media was replaced on days 1, 3 and 5 post-seeding with media containing 50 μM z-VAD-fmk (or 0.25% DMSO as a negative control). Cell pellets were obtained on day 6 post-seeding for nucleic acid extraction as described above for acutely infected cells.

For apoptotic cell labeling with FITC-labeled z-VAD-fmk, persistently infected cell lines were cultured for 5 days (37 °C or 33 °C) in growth media. Cells were incubated at the respective temperatures for the final 4 h of the culture period in fresh culture media containing 2% (HM175/clone 1 infected A549 cells) or 1% (HM175/clone 1 infected FRhK-4 cells) heat-inactivated FBS, and 10 μM FITC-labeled z-VAD-fmk (Promega Corp). Cell cultures were then washed twice with room temperature PBS, fixed with cold methanol in PBS (80% methanol:20% PBS, vol:vol) at 4 °C for 30 min, and washed twice with room temperature PBS. Nuclei were counter stained with DAPI using SlowFade® Gold (Invitrogen, Molecular Probes, Carlsbad, CA) according to manufacturer's instructions. Parental cell lines (i.e., uninfected) were similarly cultured, treated with labeled z-VAD-fmk, and subjected to post-labeling treatment prior to fluorescent microscopy as for the persistently infected cell lines.

Light and fluorescent microscopy

At given days post-infection of acute or mock infection, or post-seeding of persistently infected or uninfected cells, cell cultures were observed and digital image photography completed using brightfield and/or phase contrast microscopy with a Ziess Axiovert 25 microscope and LCD camera using Axiovision4 software (Carl Zeiss, Inc., Thornwood, NY). A Nikon Eclipse TE2000-S microscope and digital camera in conjunction with Spot 4.7 software package (Diagnostic Instruments, Inc., Sterling Heights, MI) was used for fluorescent microscopy and digital image photography of FITC-z-VAD-fmk treated cell cultures.

RNA isolation, rRNA degradation and DNA laddering analysis, and protein extracts

Total cytoplasmic RNA was isolated for the analysis of rRNA degradation and DNA laddering by agarose gel electrophoresis as described previously (Goswami et al., 2004; Kulka et al., 2003). Similarly extracted RNA was also used for the analysis of viral negative-strand synthesis by RT-qPCR. RNA concentrations were calculated from the absorbance measured at 260 nm. Protein extracts were obtained as total cell (soluble) lysates from mock or virus infected cells using RIPA buffer (plus protease and phosphatase inhibitors) as previously described (Kulka et al., 2003, 2009). Fractions were stored in aliquots at −70 °C. Protein concentrations were determined using the BCA-200 Protein Assay (Pierce) according to the manufacturer's instructions.

Reverse transcription and quantitative PCR

Total cytoplasmic RNAs from infected or mock-infected cells were subjected to DNase I digestion, phenol:chloroform:isoamyl alcohol (Invitrogen) extraction and ethanol precipitation prior to reverse transcription (1 μg RNA per reaction) with AMV reverse transcriptase (RT) using the following primer sequences: HAV sense: 5′ CCGTTTGCCTAGGCTATAGGCTA 3′; HAV anti-sense: 5′ CAGCTCCATGC TAATCATGGAGT 3′ to detect the presence of viral negative strand RNA as previously described (Kulka et al. 2009). PCR primers were obtained from Loftstrand Labs, Ltd (Gaithersburg, MD). Following reverse transcription, qPCR amplification was performed in the Applied Biosystems 7900 HT. Briefly, real-time PCR was performed using 96 well plates containing sample cDNA (2.5 μl) in a 50 μl volume with TaqMan 2X Universal PCR master mix (Applied Biosystems, manufactured by Roche, Branchburg, NJ), and forward and reverse primers (0.4 μM) and FAM-labeled probe (150 nM) were used as previously described (Jothikumar et al., 2005). Primers and probe for q-PCR were obtained from Integrated DNA Technologies, Coralville, IA. All samples were tested in duplicate, with a sample containing no template as negative control. The thermal cycler profile was as follows: 50 °C for 2 min, 95 °C for 10 min, then 95 °C for 15 s, 60 °C for 1 min for 40 cycles, followed by 1 h 20 min at room temperature. PCR products were analyzed by agarose gel electrophoresis (Goswami et al., 2004).

PAGE and western blot analyses

Equal amounts of protein (RIPA) extracts were adjusted to equivalent volumes in denaturing sample buffer containing 100 mM DTT, heated at 95–98 °C for 5–10 min and subjected to SDS-PAGE [4–12% Bis-Tris gels using MOPS-SDS running buffer (Invitrogen)] under reducing conditions, followed by semi-dry electrophoretic transfer onto nitrocellulose membranes using a three buffer transfer system based on manufacturer's instructions (Owl Separation Systems, Portsmouth, NH). Western blot analysis

was performed as previously described for antibodies to HAV VP1, caspase-3, and actin (Kulka et al., 2003, 2009; Goswami et al., 2004) and primary antibodies were diluted (anti-VP1 and anti-actin at 1:2000; anti-caspase-3 at 1:1000) in TBS-T containing 5% nonfat dry milk (NFDM) for overnight incubation at 4 °C. Secondary antibody-HRP conjugates were used at a 1:10,000 dilution in TBS-T/5% NFDM for 1 h incubation at RT and subsequent detection by chemiluminescence was as previously described (Kulka et al., 2003).

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